FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **MUR-7450**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5): **TO BE ASSIGNED** 09/142095

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| **PCT/GB97/00577** | **03 MARCH 1997 (03.03.97)** | **01 MARCH 1996 (01.03.96)** |

TITLE OF INVENTION: **DRUG TRIAL ASSAY SYSTEM**

16 MARCH 1996 (16.03.96)

APPLICANT(S) FOR DO/EO/US: **BURCHELL, Brian**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). (UNSIGNED)
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

**Items 13 to 18 below concern document(s) or information included:**

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

300 Rec'd PCT/PTO 01 SEP 1998

| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| **TO BE ASSIGNED** | **PCT/GB97/00577** | **MUR-7450** |

20. The following fees are submitted:.

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5)) :**

| | | CALCULATIONS PTO USE ONLY |
|---|---|---|
| ☒ Search Report has been prepared by the EPO or JPO . . . . . | **$930.00** | |
| ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) . . . . . | **$720.00** | |
| ☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) . . . . . | **$790.00** | |
| ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO . . . . . | **$1,070.00** | |
| ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) . . . . . | **$98.00** | |

| | | |
|---|---|---|
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | **$930.00** | |
| Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). | **$0.00** | |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
|---|---|---|---|---|---|
| Total claims | 14 - 20 = | 0 | x $22.00 | **$0.00** | |
| Independent claims | 1 - 3 = | 0 | x $82.00 | **$0.00** | |
| Multiple Dependent Claims **(check if applicable)**. | | | ☐ | **$0.00** | |
| **TOTAL OF ABOVE CALCULATIONS** | | | = | **$930.00** | |
| Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. | | | ☐ | **$0.00** | |
| **SUBTOTAL** | | | = | **$930.00** | |
| Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). | | | + | **$0.00** | |
| **TOTAL NATIONAL FEE** | | | = | **$930.00** | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** | | | ☐ | **$0.00** | |
| **TOTAL FEES ENCLOSED** | | | = | **$930.00** | |
| | | | | **Amount to be: refunded** | $ |
| | | | | **charged** | $ |

☒ A check in the amount of **$930.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **18-0350** A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

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REGISTRATION NUMBER

**01 September 1998**
DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| Applicant: Brian Burchell | : Art Unit: |
| Serial No.: TO BE ASSIGNED | : Examiner: |
| Filed: HEREWITH | : |
| FOR: DRUG TRIAL ASSAY SYSTEM | : |
| | : |

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents
Washington, D.C. 20231

S I R :

Prior to examination of the above-identified application, please amend the above application as follows:

IN THE SPECIFICATION:

On page 1, before the title, please insert the following:

--This application is the U.S. National Phase of PCT International Application No. PCT/GB97/00577.--.

IN THE CLAIMS:

Please amend the claims 3, 4, 5, 6, 7, 8, 10, 11, 12, and 14 as follows:

3. Use of a test as claimed in claim 1 [or 2] wherein the sample is chosen from blood, buccal smear or any other sample containing DNA from the potential participants.

4. Use of a test as claim in [any of the preceding] claim[s] 1 further comprising the step of eliminating participants having the genetic basis of Gilbert's Syndrome from a drugs trial.

5. Use of a test as claimed in [any of] claim[s] 1 1[to 3] wherein the method comprises the further step of selecting only participants having genetic basis for Gilbert's Syndrome for a drugs trial.

6. Use of a test claimed in [any of] claim[s]s 1 [to 3] further comprising the step of interpreting the results of the drugs trial in the knowledge that certain participants have Gilbert's Syndrome.

7. Use of a test as claimed in [any of the preceding] claim[s] 1 wherein the method comprises the steps of:

a) isolating DNA from each sample,

b) amplifying the DNA inner region indicating the genetic basis for Gilbert's Syndrome,

c) isolating amplified DNA fragments, and

d) identifying individuals having the genetic basis of Gilbert's Syndrome.

8. Use of a test as claimed in [any of the preceding] claim[s] 1 wherein the DNA is amplified using the polymerase chain reaction (PCR) using a radioactively labeled pair of nucleotide primers.

10. Use of a test as claimed in [any of] claim[s] 7 [to 9] wherein the DNA region indicating the genetic basis of Gilbert's Syndrome is the gene encoding UDP-glucuronosyltransferase (UGT).

11. Use of a test as claimed in [any of] claim[s] 7 [to 10] wherein the DNA to be amplified is in an upstream promoter region of the UGT 1*1 exon 1.

12. Use of a test as claimed in [any of] claim[s] 7 [to 11] wherein the DNA to be amplified includes the regions between –35 and –55 nucleotides at the 5' end of UGT 1*1 exon.

14. Primers for use of a test as claimed in [any of the preceding] claim[s] 1 including primer pairs, AB or CD as follows:

A/B(1,5' -AAGTGAACTCCCTGCTACCTT-3',
B,5' -CCACTGGGATCAACAGTATCT-3') or
C/D (C,5'-GTCACGTGACACAGTCAAAC-3';
D 5' -TTTGCTCCTGCCAGAGGTT-3').

Respectfully Submitted,

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AR:sls

Dated: September 1, 1998

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The Assistant Commissioner for Patents is hereby authorized to charge payment to Deposit Account No. **18-0350** of any fees associated with this communication.

**EXPRESS MAIL** Mailing Label Number: EL 013 897 799 US
Date of Deposit: September 1, 1998

I hereby certify that this paper and fee are being deposited, under 37 C.F.R. § 1.10 and with sufficient postage, using the "Express Mail Post Office to Addressee" service of the United States Postal Service on the date indicated above and that the deposit is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231, Attn: Box PCT (RO/US)

Jan Landis

4PRTS

09/142095

WO 97/32042

PCT/GB97/00577

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"Drug Trial Assay System"

The present invention relates to drug trials, usually carried out for or on behalf of pharmaceutical companies. More particularly the invention relates to a method for improving the efficacy of drug trials.

In the different stages of drug trials, regulatory authorities in different European countries and the FDA in the USA require extensive data to be provided in order to approve use of the drugs.

It is important that as much information as possible is available in relation to all participants who take part in drug trials, from volunteers who take part in phase 1 trials to patients involved in stage 3 clinical trials.

In particular, if certain individuals or groups of individuals have severe or abnormal reactions to drug administration, further studies involving that drug will be in jeopardy unless the reason for the reaction is realised.

The knowledge of pharmacogenetics can play an important

role in understanding the impact of drug metabolism on pharmacokinetics, role of receptor variants in drug response and in the selection of patient populations for clinical studies.

Considerable effort has been expended in attempting to identify the pharmacogenetic basis of idiosyncratic adverse drug reactions, particularly hypersensitivity reactions. While there is clear evidence for pharmacogenetic influence on susceptibility to hypersensitivity reactions, necessary and sufficient pharamacogenetic defects have not been identified.

The clinical implications of genetic polymorphism in drug metabolism have been studied extensively (See Tucker GT (1994) Journal Pharamacology 46 pages 417-424).

Gilbert's Syndrome (GS) is a benign unconjugated hyperbilirubinaemia occurring in the absence of structural liver disease and overt haemolysis and characterized by episodes of mild intermittent jaundice. It is part of a spectrum of familial unconjugated hyperbilirubinaemias including the more severe Crigler-Najjar (CN) syndromes (types 1 and 2). GS is the most common inherited disorder of hepatic bilirubin metabolism occurring in 2-12% of the population and is often detected in adulthood through routine screening blood tests or the fasting associated with surgery/intercurrent illness which unmasks the hyperbilirubinaemia[1-3]. The most consistent feature in GS is a deficiency in bilirubin glucuronidation but altered metabolism of drugs has also been reported[3-5]. Altered rates of bilirubin production, hepatic haem production and altered hepatic uptake of bilirubin have been reported in some GS patients[2].

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Due to the benign nature of the syndrome and its prevalence in the population it may be more appropriate to consider GS as a normal genetic variant[2] exhibiting a reduced bilirubin glucuronidation capacity (which in certain situations such as fasting, illness or administration of drugs) could precipitate jaundice.

In drug trials where high levels of serum total bilirubin is detected for certain individuals, it is not clear whether this is because the individuals have Gilbert's Syndrome or if it because of an effect of the drug. Whereas presently, results are explained merely by saying that the individuals have Gilbert's Syndrome, it is suspected that in the future, it will be necessary to prove this fact.

Where a jaundiced phenotype is apparent after volunteers have been accepted for a trial and have been subjected to five days of a strict diet, no alcohol and no smoking, the jaundiced appearance giving an indication that the individuals have Gilbert's Syndrome, may cause them to be ruled out of the trials. Therefore, where approximately 250 individuals would be required for phase 1 trials and about 6000 patients for phase 3 trials, unnecessary time and effort would have been spent during the first 5 days of these trials and individuals having Gilbert's Syndrome may be ill effected.

Bosma et al. (New England Journal of Medicine (1995) volume 333 Number 18) reported the genetic basis of Gilbert's syndrome.

The present invention aims to provide a method of improving the efficacy of drug trials in view of the problems mentioned above.

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According to the present invention there is provided a method for improving the efficacy of drug trials, the method comprising the step of screening samples from

individuals for the genetic basis of Gilbert's Syndrome.

In a prefered embodiment of the invention the method comprises the steps taking a sample from each potential participant in a drug trial, screeing the samples for the genetic basis of Gilbert's Syndrome, identifying participants having the genetic basis of Gilbert's Syndrome.

The sample may comprise blood, a buccal smear or any other sample containing DNA from the individual to be tested.

In one embodiment the method comprises the further step of eliminating participants having the genetic basis of Gilbert's Syndrome from the drug trial.

In an alternative embodiment, the method can comprise the further step of selecting participants having the genetic basis of Gilbert's syndrome and eliminating others from the drug trial.

In a further alternative the results of the drug trials can be interpreted in the knowledge that certain participants have Gilbert's Syndrome.

Preferably the method comprises the steps of isolating DNA from each sample, amplifying the DNA in a region indicating the genetic basis of Gilbert's Syndrome, isolating amplified DNA fragments by gel electrophoresis and identifying individuals having the genetic basis of Gilbert's disease.

Preferably the DNA is amplified using the polymerase chain reaction (PCR) using a radioactively labelled

pair of nucleotide primers.

The primers are designed to prime the amplification reaction at either side of an area of the genome known to be associated with Gilbert's Syndrome.

Preferably the DNA region indicating the genetic basis of Gilbert's Syndrome is the gene encoding UDP-glucuronosyltransferase (UGT).

By gene is meant, the non coding and coding regions and the upstream and downstream noncoding regions.

In a preferred embodiment the DNA to be amplified is in an upstream promoter region of the UGT1*1 exon1.

Most preferably the DNA to be amplified includes the region between -35 and -55 nucleotides at the 5' end of UGT1*1 exon.

According to the invention there are provided suitable primers for use in a PCR reaction including primer pairs;

A/B(A,5'-AAGTGAACTCCCTGCTACCTT-3',
B,5'-CCACTGGGATCAACAGTATCT-3') or
C/D (C,5'-GTCACGTGACACAGTCAAAC-3';
D 5'-TTTGCTCCTGCCAGAGGTT-3')

The invention further comprises a kit for screening individuals for participation in drug trials, the kit comprising primers for amplifying DNA in a region of the genome indicating the genetic basis of Gilbert's Syndrome.

Using primer sequences as described herein, DNA can be amplified and analysed using among others any of the following protocols;

Protocol 1 Radioactive method

1. Extract DNA from Buccal Cells or 3ml Blood.

2. Choose primers from either side of the "TATA" box region of UGT1*1 exon1 regulatory sequence. Freshly end label one primer with [$\gamma$ $^{32}\alpha$]-ATP (40 min).

3. Amplifying a small region up to 100 bp in length by PCR (2h).

4. Apply to 6% PAG denaturing gel (preparation, loading, run time, 4h).

5. Expose (-70°C) wet gel to autoradiographic film (15 min).

This method takes about 7h to complete. Polymorphisms only observed in TATA box non coding region todate.

Protocol 2
Alternative Radioactive Method: Solid Phase Minisequencing

1. Extract DNA (as above)

2. Prepare primers biotinylating one

3. Amplify DNA by PCR using primers

4. Captive biotinylated PCR products on streptavidin coated support and deactive.

5. Carry out primer extension reaction sequencing.

Protocol 3
Non-Radioactive Methods:

(a) Analysis by Single Strand Conformational Polymorphism (SSCP)

1. Extract DNA (as above).

2. Choose primers either side of the TATA Box.

3. Amplify a small region up to 100 bp in length by PCR (2H).

4. Denature and place on ice (15 min).

5. Load onto a non-denaturing PAG gel, (preparation/load/run time, 4h).

6. Stain with Ethidium bromide or silver nitrate (30 mm).

This method still takes about 7h to complete, but is potentially slightly cheaper since there is no radioactivity or autoradiography.

This method could be done on an automated DNA sequencer from stage 5, if primers are tagged with chromophores in PCR stages 2 and 3. Result would then be read automatically.

(b) Oligonucleotide Assay Hybridization

1. Extract DNA (as above).

2. Choose primers and amplify DNA by PCR up to 100 bp in length.

3. Apply DNA to plastic grids.

4. Screen bound DNA samples with specific DNA probes for $TA_5$, $TA_6$, $TA_7$ tagged with different coloured/fluorescent chromphores.

5. Read ouput automatically for experimental protocols.

References

Monaghan G et al. Lancet (1996) 347 578-581.

"Detection of polymorphisms of human DNA by gel electrophoresis or single-strand conformational polymorphisms"." Orita M et al. Proc Matl Acad Sci (USA) (1989) 86 2766-2700.

"Assays of complementary oligonucleotides for analysing Hybridization behaviour of Nucleic Acids". Southern E M. Nuc Acids Res (1194) 22 1368-1373.

The basis of the invention is illustrated in the following example with reference to the accompanying figures wherein:

Figure 1 illustrates genotypes at the TATA box sequence upstream of the UGT1*1 exon 1 determined by direct sequencing and radioactive PCR.

Figure 2 illustrates serum total bilirubin (μmol/l) plotted against UGT1*1 exon 1 genotype.

Figure 3 illustrates segregation of the 7/7 genotype with elevated serum total bilirubin concentration in a family with GS.

Figure 4 illustrates the 5′ sequence of the UGT1*1 exon 1 and the position of the primers with respect to the UGT gene.

Example

We have examined the variation in the serum total bilirubin (STB) concentration in a representative group of the Eastern Scottish population (drug-free, alcohol-free non-smokers) in relation to genotype at the UDP-glucuronosyltransferase subfamily 1 (UGT1) locus. Subjects with the 77/7 genotype in this population have a significantly higher STB than those with 6/7 or 6/6 genotypes. Of 14 control subjects who underwent a 24 hour fast to establish whether they had Gilbert Syndrome (GS), only 7/77 subjects had GS. In addition, one confirmed GS patient, two recurrent jaundice patients and 9 clinically diagnosed GS patients had the 7/7 genotype. Segregation of the 7/7 genotype with elevated STB concentration has also been demonstrated in a family of 4 Gilbert members. This incidence of

the 7/7 genotype in the population is 10-13%. Here, we demonstrate a correlation between variation in the human STB concentration and genotype at a TATA sequence upstream of the UGT1*1 exon 1 and that the 7/7 genotype is diagnostic for GS.

The inheritance of GS has been described as autosomal dominant or autosomal dominant with incomplete penetrance based on biochemical analysis[6]. More recent reports have suggested that the mildly affected (Gilbert) members of families in which CN type 2 (CN-2) occurs are heterozygous for mutations in the UDP-glucuronosyltransferase subfamily 1 (UGT1) gene which cause CN-2 in the homozygous state. The inheritance of GS in these families is autosomal dominant while CN-2 is autosomal recessive[7-11]. However, the incidence of CN-2 in the population is rare and the frequency of alleles causing CN-2 would not be sufficient to explain the population incidence of GS.

An abstract by Bosma et al[12] suggested a correlation between homozygosity for a 2bp insertion in the TATA box upstream of UGT1*1 exon 1 and GS (no mutations were found in the coding sequence of the UGT1*1 gene). In this report we demonstrate that the primary genetic factor contributing to the variation in the serum total bilirubin (STB) concentration in the Eastern Scottish population is the sequence variation reported by Bosma et al[12]. In addition, we show that the 7/7 genotype is associated with GS and occurs in 10-13% of the population.

Methods

Patients and Controls

Whole blood (3ml) was collected into EDTA(K3) Vacutainer tubes (Becton Dickinson) from one confirmed

male Gilbert patient (diagnosed following a 48 hour restricted diet[13]), two female patients with recurrent jaundice/associated elevated STB (29-42 μmol/1) and 9 (1 female, 8 male) clinically diagnosed GS subjects (persistent elevation of the STB amidst normal liver function tests.) The patients were aged 22-45 years.

77 non-smoking residents selected at random from the Tayside/Fife region of Scotland (39 females aged 19-58 years, mean 32.41± 10.94; 38 males aged 23-57, means 35.58 ± 9.04) participated in this study. Whole blood (9ml) was collected 8-10am) into EDTA(K3) Vacutainer tubes (Becton Dickinson) for DNA extraction and SST Vacutainer tubes (Becton Dickinson) for biochemical investigations. The subjects had not taken any medication or alcohol in the previous 5-7 days and had fasted overnight (12 hours). 14 controls subsequently underwent further biochemical tests (following a 3 day abstinence from alcohol) before and after a 24 hour 400-calorie diet[14] to determine if they had GS. All patients/controls were fully informed of the study and gave consent for their blood to be used in this study.

Biochemistry and DNA Extraction

The following biochemical tests were performed on control blood samples; alanine aminostransferase, albumin, alkaline phosphatase, amylase, STB, cholesterol, creatinine, creatine kinase, free thyroxine, gamma-glutamyl-transferase, glucose, HDL-cholesterol, HDL-cholesterol/total cholesterol, iron, lactate dehydrogenase, percentage of saturated transferrin (PSAT), proteins, serum angiotensin converting enzyme, thyroid stimulating hormone, transferrin, triglycerides, urate, urea. 14 controls also had pre- and post-fasting (24 hour) alanine

aminostransferase, albumin, alkaline phosphatase, STB and urate measured. DNA was prepared using the Nucleon II Genomic DNA Extraction Kit (Scotlab) according to manufacturer's instructions.

Genotyping

Polymerase Chain Reaction

Primer pairs A/B (A, 5'-AAGTGAACTCCCTGCTACCTT-3'; B, 5'-CCACTGGGATCAACAGTATCT-3') or C/D (C,5'-GTCACGTGACACAGTCAAAC-3';D, 5'-TTTGCTCCTGCCAGAGGTT-3') flanking the TATA box sequence upstream of the UGT1*1 exon 1 were used to amplify fragments of 253-255bp and 98-100bp, respectively. Amplifications (50$\mu$l) were performed in 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 50mM KCl, 10mM Tris.HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5mM $MgCl_2$, 0.25$\mu$M of each primer, 1 Unit of Taq Polymerase (Promega) and human DNA (0.25-0.5$\mu$g). The polymerase chain reaction (PCR) conditions using the Perkin-Elmer Cetus DNA Thermal Cycler were: 95°C 5 min followed by 30 cycles of 95° 30 sec, 58°C 40 sec, 72°C40 sec. 1*1

Direct Sequencing

Amplification was confirmed prior to direct sequencing by agarose gel electrophoresis. Sequencing was performed using [$\alpha$-$^{35}$S]-dATP (NEN Dupont) with the USB Sequenase™ PCR Product Sequencing Kit according to manufacturer's instructions. Sequenced products were resolved on 6% denaturing polyacrylamide gels. The dried gels were exposed overnight to autoradiographic film prior to developing.

Radioactive PCR

Amplification was performed as above using primer pair C/D except that 2.5 pmol of primer C was radioactively 5' end-labelled with 2.5μCi of [γ-$^{32}$P]-ATP (NEN Dupont) prior to amplification. Products were resolved on 6% denaturing polyacrylamide gels and the wet gels exposed to autoradiographic film (-70°C 15 min) and the autoradiographs developed.

Statistics

A t-test was used to determine if there was a significant age difference between males and females. $\chi^2$ analysis was used to assess any difference in the distribution of the 6/6, 6/7 and 7/7 genotypes in males and females and also to determine if the 7/7 subjects from the 24 hour fasted group had STB elevated into the range diagnostic for GS[14]. An analysis of variance was performed to compare mean STB in males and females within each genotype group. A non-parametric test, the Mann-Whitney U-Wilcoxon Rank Sum W Test was used to determine whether there was a significant difference in mean STB between males and females (irrespective of genotype). Correlations and significance tests were performed for STB versus PSAT and STB versus iron. A probability (p) of $< 0.05$ was accepted as significant.

Results

In Figure 1 a photographic representation of the sense DNA sequences obtained by PCR/direct sequencing of DNA samples having the genotypes 6/6, 6/7 and 7/7 is shown. The common allele, $(TA)_6TAA$, is denoted by "6" while the rarer allele, $(TA)_7TAA$, is denoted by "7". Below each sequence is an overexposed photographic representation of the 98 to 100bp resolved fragments amplified using primer pair C/D which flank the TATA sequence upstream

of the UGT1*1 exon 1. The additional fragments of 99 and 101 bases are thought to be artifacts of the PCR process where there is non specified addition of an extra nucleotide to the 3' end of the amplified product[21]. Figures 1b illustrates results after testing a range of unknown individuals.

In Figure 2 males (M) and females (F) are plotted separately. Each circle/square represents the result of a single control subject. The squares indicate the 14 controls who also underwent the 24 hour restricted diet (see Methods). The filled circles/squares represent those who had a lower than normal PSAT (≤ 22%) while the half-tone circles represent those who had a higher than normal PSAT (≥ 55%). The mean STB concentrations (indicated by the horizontal lines) for males were 13.24 ± 3.88 (6/6), 13.94 ± 6.1 (6/7) including control h or 12.69 ± 3.34 excluding control h, 29 ± 14.45 (7/7) and for females were 9 ± 3.62 (6/6), 12.2 ± 3.53 (6/7), 21.6 ± 7.8 (7/7). The encircled result is from control h (discussed in the text).

In Figure 3 males and females are represented by squares and circles, respectively. Filled and half-filled circles/squares indicate the genotypes 7/7 and 6/7, respectively. The numbers in parentheses below each member of the pedigree are the STB concentrations measured after a 15 hour fast and 7 day abstinence from alcohol. All family members were non smokers who were not taking any medication when the biochemical tests were performed. Elevated STB are underlined. Individual members of each generation (I or II) are denoted by the numbers 1-4 above each circle/square. Generation III have not yet been tested.

There was no significant age difference between males and females ($t = -1.38$, $p = 0.17$). Genotypes were determined initially by amplification/sequencing and later by the radioactive PCR approach. Individuals homozygous for the common allele, hetrozygous or homozygous for the rarer allele have the genotypes 6/6, 6/7 and 7/7, respective. 12 DNA samples (2 of 6/6, 3 of 6/7 and 4 of 7/7) were analysed by both methods and genotype results were identical (see Figure 1).

Genotype frequencies in male controls were 6/6 (44.74%, 6/7 (44.74%), 7/7 (10.52%) and in female controls were 6/6 (35.9%), 6/7 (51.3%), 7/7 (12.8%). There was no significant difference between the genotype proportions in the two groups ($\chi^2 = 0.6$ at 2 df, $p = 0.7$). Control h (encircled in Figure 2) had a STB which was 2.4 SD above the mean STB for that group (mean calculated including control h). The results for control h were repeatable and he is currently being investigated to exclude haemochromatosis. Comparison of mean STB in males and females revealed that females have a significantly lower concentration than males ($p = 0.031$ including control h; $p + 0.0458$ excluding control h). There was a strong correlation between genotype and mean STB concentration within the control group ($p < 0.001$) irrespective of whether control h was included and there was a significant difference in mean STB between males and females of the same genotype ($p < 0.05$) irrespective of whether control h was included (see Figure 2). All patients studied had the 7/7 genotype.

Correlations between STB/PSAT ($r = 0.4113$, $p = 0.001$)(see Figure 2) and STB/iron females ($p = 0.001$) than males ($p = 0.01$) but when control h is excluded there was no significant correlation in males.

The STB concentrations of control who underwent the 24 hour restricted diet (see Methods) are shown in Table 1. The normal fasting response is a small rise in the base-line STB (not exceeding a final concentration of 25μmol/1) most of which is unconjugated while GS patients have a lone biochemical feature a raised STB ()25μmol/1 but ⟨50μmol/1) most of which is unconjugated[14]. The 6/6 and 6/7 controls had post-fasting STB of ≤23μmol/1 while all 7/7 controls were ≥31μmol/1. Other liver function tests were within acceptable ranges for the age and sex of the subjects. The 7/7 genotype correlates with a fasted STB (24 hour) within the range diagnostic for GS[14] (p ⟨ 0.01)(see Table 1). In addition, the 7/7 genotype segregates with elevated STB concentration in a family with 4 GS members (Figures 3).

Table 1 shows a comparison of the UGT1*1 exon 1 genotype with elevation in the serum total bilirubin after a 24 hour 400-calorie restricted diet[14].

An elevation of the fasting STB to a final concentration in the range 25-50μmol/1 is considered to be diagnostic for GS[14]. The 7/7 subject denoted by * has a fasting and non-fasting STB of ⟩ 50μmol/1 but this value is within a range considered by others to conform to a diagnosis of GS[7-11].

**Table 1**

| Genotype | Sex | 24 hour fast | | Fasting bilirubin >25 & <50μmol/l |
|---|---|---|---|---|
| | | Before | After | |
| 6/6 | M | 8 | 17 | NO |
| | M | 9 | 19 | NO |
| | M | 12 | 15 | NO |
| 6/7 | F | 8 | 17 | NO |
| | F | 9 | 13 | NO |
| | F | 11 | 12 | NO |
| | F | 12 | 17 | NO |
| | M | 8 | 10 | NO |
| | M | 15 | 23 | NO |
| | M | 17 | 18 | NO |
| 7/7 | F | 9 | 34 | YES |
| | F | 12 | 34 | YES |
| | M | 19 | 31 | YES |
| | M | 62 | 96 | NO* |

Discussion

A few recent reports claim to have identified the genetic cause of GS[10-12]. Clinical diagnosis of GS is often based on a consistent midly elevated non-fasting STB (⟩17 μmol/1) as the sole abnormal liver function test, intermittent jaundice or both. The diagnosis can be confirmed by elevation of the STB to 25-50μmol/1 after a 24 hour 400-calorie diet[14] or by elevation of the unconjugated bilirubin by ⟩ 90% within 48 hours of commencing a 400 calorie diet[13].

Sato's research group recently reported the occurrence of 7 different heteroxygous missence mutations in unrelated Gilbert patients (most of the mutations have been found in the homozygous state in affected members of CN families), however, the non-fasted STB for these patients were ⟩ 52μmol/1 (with the exception of one,

31μmol/l)[10,12]. These non-fasted STB concentrations already exceed the diagnostic range for GS[14], hence these patients have a more severe form of hyperbilirubinaemia than those studied in this report, while those in the Bosma et al[12] abstract had STB concentrations similar to those studied here.

The example herein shows that the variation in the [illegible] levels after an overnight fast (and in the absence of exposure to known inducers of the UGT1*1 isoform in GS, such as alcohol [15] and drugs[16]) a representative group of the Eastern Scottish population is primarily due to (or associated with) the TATA box sequence variation reported by Bosma et al[12]. In agreement with previous work females have a significantly lower mean STB concentration than males[17-18].

Individuals with the 7/7 genotype in the population have GS (see Table 1). One of the 7/7 controls indicated in Table 1 had a non-fasting STB similar to those reported for heterozygous carriers of CN-2 mutations[7-11] which suggests that this subject may also be a carrier of a CN-2 mutation, alternatively, the very elevated bilirubin in this patient may be due to the coexistence of Reavon's Syndrome (characterized by a collection of abnormal biochemical results which are risk factors for coronary heart disease)[19].

We have found that 10-13% of the Eastern Scottish population have the genotype associated with mild GS. None of the Gilbert subjects from the control population were aware that they had an underlying metabolic defect in glucuronidation with testifies to its benign nature. Three 7/7 controls had STB concentrations comparable to mean levels observed in heterozygotes, however, they also had a lower than

normal PSAT (≤22%)(see Figure 2). The observed correlation between STB and PSAT ($p = 0.001$) (Figure 2) and STB and iron (females $p = 0.001$ and males $p = 0.01$ including control h) indicates that other genetic and environmental factors affecting the serum PSAT and iron values will in turn affect the STB concentration.

From the data presented here and previous reports it seems clear that there are mild and more severe forms of GS. The milder form (fasted STB 25-50μmol/l) is either caused by (or is associated with) a homozygous 2bp insertion at the TATA sequence upstream of the UGT1*1 exon 1 (autosomal recessive inheritance) while the rarer more severe dominantly inherited forms identified to date[7-11] (non-fasted STB ⟩ 50μmol/l are due to heterozygosity for a mutation in the coding region of the UGT1*1 gene which in its homozygous state causes CN-2. The particular genetic abnormality causing GS in a patient will have implications for genetic counselling as the dominantly inherited form of two GS patients could result in offspring with CN-2, whereas the recessive form in one or both GS patients would have less serious implications. It is important to discriminate between the two forms and provide suitable genetic counselling for such couples. The rapid DNA test presented here (less than 1 day for extracted DNA) carried out in addition to biochemical tests following a 12 hour overnight fast (without prior alcohol or drug intake would permit such a diagnosis. The compliance rate for the current 24 and 48 hour restricted diet tests for GS[13-14] is debatable and hence the overnight fast has obvious advantages and only one blood sample or a buccal smear is required (for genetic and biochemical analysis) in contrast to the 2-3 blood samplings required for the 24 and 48 hour tests. This approach to GS testing would be cost effective in terms

of fewer patient return visits to clinics and in identifying couples at risk of having children with CN-2.

In addition, the recent finding of an increased bioactivation of acetominophen (a commonly used analgesic which is eliminated primarily by glucuronidation) in GS patients indicates the greater potential for drug toxicity in these patients if administered drugs which are also conjugated by UGT1 isoforms[3]. In fact, ethinylestradiol (EE2) has recently been shown to be primarily glucuronidated by the UG[illegible] isoform in man[20] and hence this could have implications for female Gilbert patients taking the oral contraceptive who are then more predisposed to developing jaundice.

The tests outlined herein have obvious implications for setting up drug trials in understanding unusual results in ruling out individuals who may be adversely affected by the drugs or in positively choosing these individuals to determine the effects of particular drugs on hyperbilirubinaemia.

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Art 34



**CLAIMS**

1. Use of a test for detecting the genetic basis of Gilbert's Syndrome in a method to improve the efficacy of drug trials, the method comprising screening samples from potential participants for the basis of Gilbert's Syndrome and eliminating or including potential participants in a drug trial in the knowledge of them possessing or not possessing the genetic basis of Gilbert's Syndrome.

2. Use of a test as claimed in claim 1 wherein the method comprise the steps of:

   a) taking a sample from each potential participant in a drug trial,

   b) screening the samples for the genetic basis of Gilbert's Syndrome,

   c) identifying participants having the genetic basis of Gilbert's Syndrome, and

   d) proceeding with drugs trials in the knowledge of participants possessing or not possessing the genetic basis of Gilbert's Syndrome.

3 Use of a test as claimed in claim 1 or 2 wherein the sample is chosen from blood, buccal smear or any other sample containing DNA from the potential participants.

4. Use of a test as claimed in any of the preceding claims further comprising the step of eliminating participants having the genetic basis of Gilbert's

Syndrome from a drugs trial.

5. Use of a test as claimed in any of claims 1 to 3 wherein the method comprises the further step of selecting only participants having genetic basis for Gilbert's Syndrome for a drugs trial.

6. Use of a test as claimed in any of claims 1 to 3 further comprising the step of interpreting the results of the drugs trial in the knowledge that certain participants have Gilbert's Syndrome.

7. Use of a test as claimed in any of the preceding claims wherein the method comprises the steps of:

a) isolating DNA from each sample,

b) amplifying the DNA inner region indicating the genetic basis for Gilbert's Syndrome,

c) isolating amplified DNA fragments, and

d) identifying individuals having the genetic basis of Gilbert's Syndrome.

8. Use of a test as claimed in any of the preceding claims wherein the DNA is amplified using the polymerase chain reaction (PCR) using a radioactively labelled pair of nucleotide primers.

10. Use of a test as claimed in any of claims 7 to 9 wherein the DNA region indicating the genetic basis of Gilbert's Syndrome is the gene encoding UDP-glucuronosyltransferase (UGT).

11. Use of a test as claimed in any of claims 7 to 10

wherein the DNA to be amplified is in an upstream promoter region of the UGT 1*1 exon 1.

12. Use of a test as claimed in any of claims 7 to 11 wherein the DNA to be amplified includes the regions between -35 and -55 nucleotides at the 5' end of UGT 1*1 exon.

13. A kit for screening individuals participation in drug trials, the kit comprising primers for amplifying DNA in the region of the genome indicating the genetic basis of Gilbert's Syndrome.

14. Primers for use of a test as claimed in any of the preceding claims including primer pairs, AB or CD as follows:

A/B(A,5'-AAGTGAACTCCCTGCTACCTT-3',
B,5'-CCACTGGGATCAACAGTATCT-3') or
C/D (C,5'-GTCACGTGACACAGTCAAAC-3';
D 5'-TTTGCTCCTGCCAGAGGTT-3').

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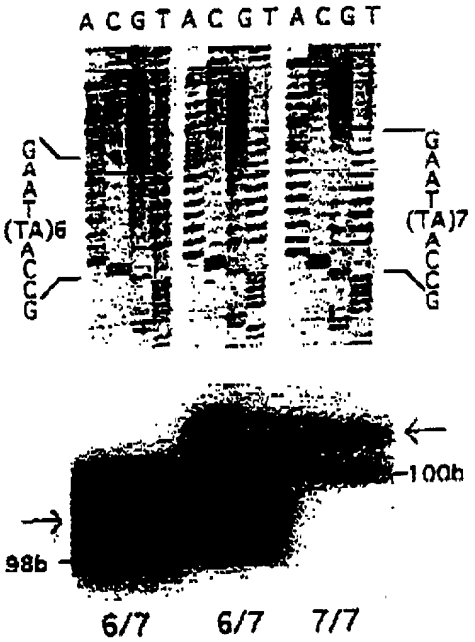


FIG. 1a


FIG. 1b

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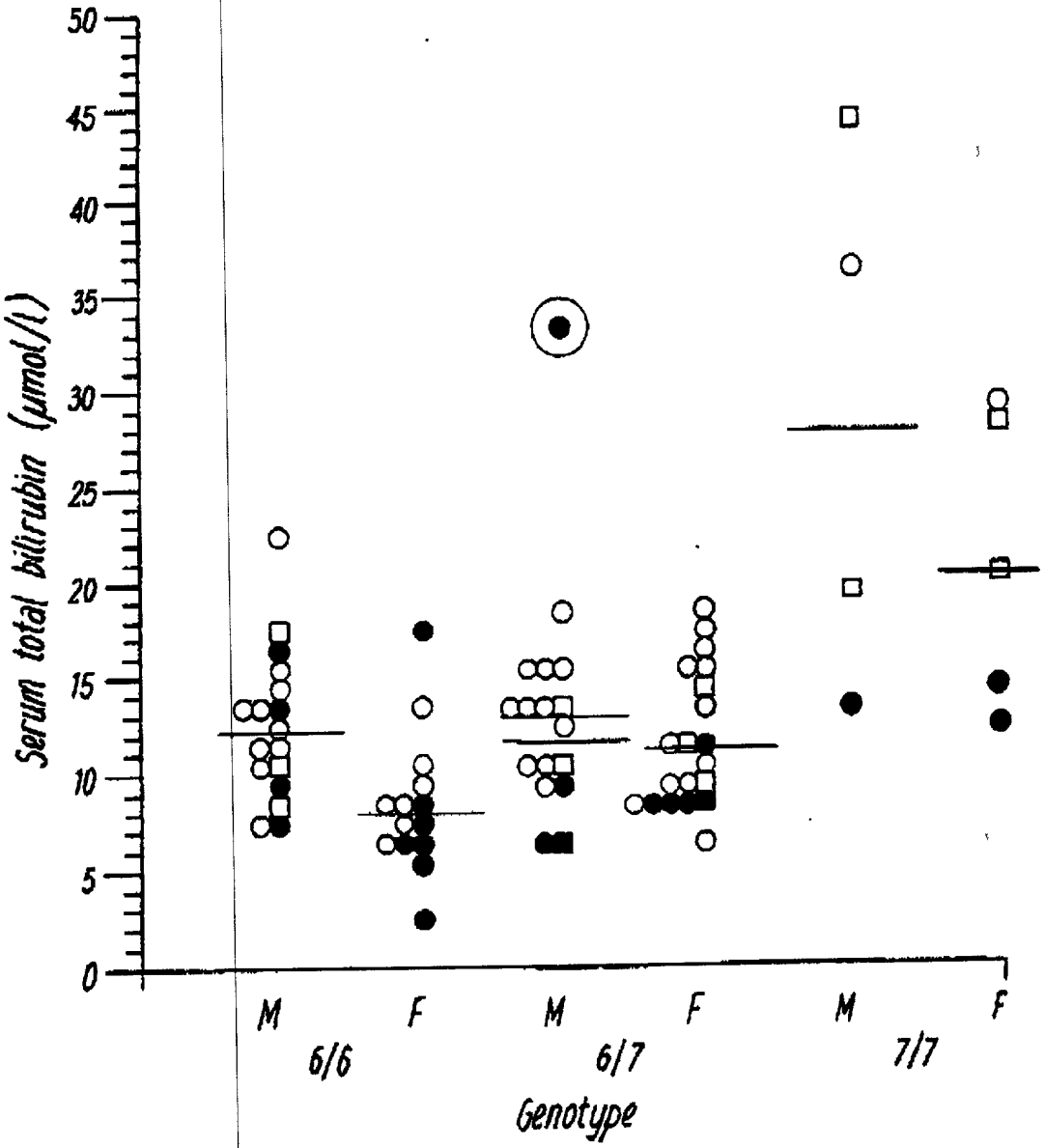
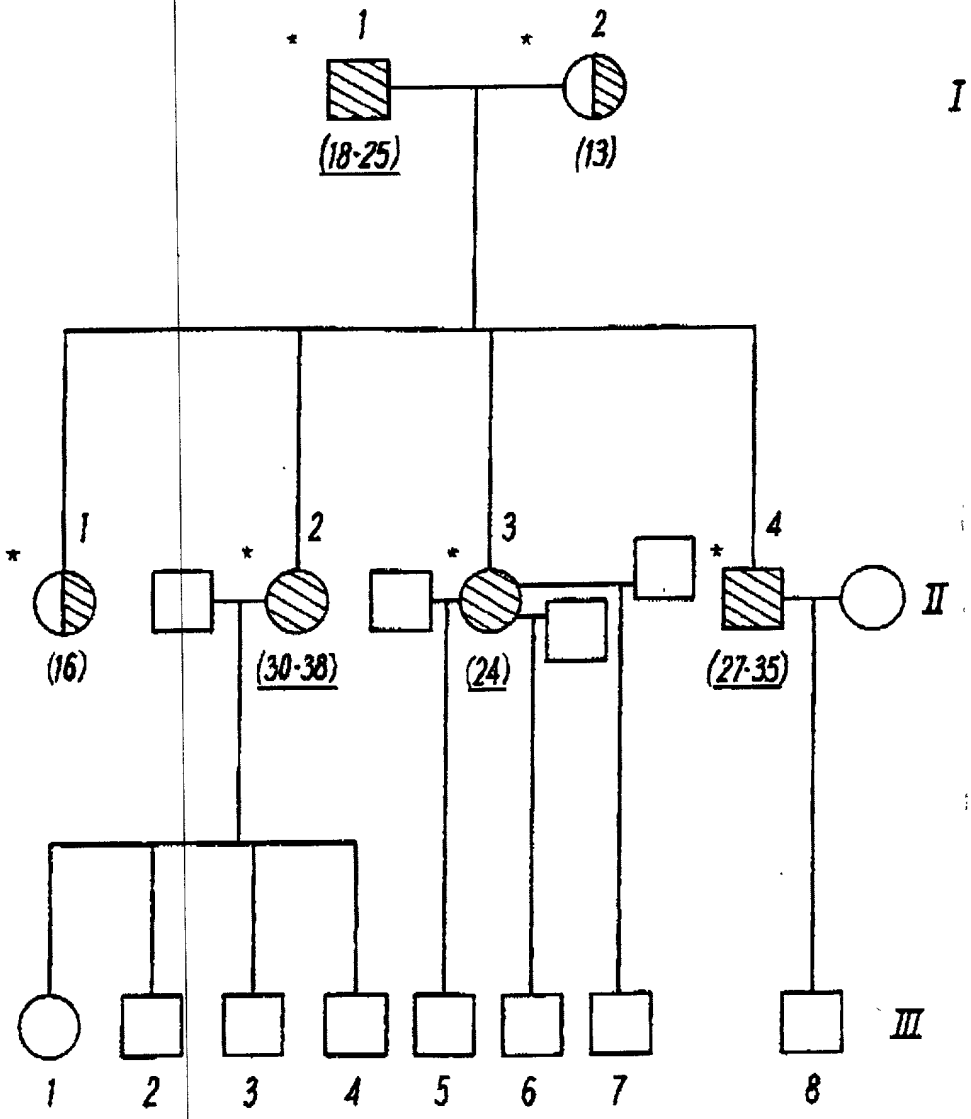



FIG.2

Pedigree Showing Segregation of the Gilbert Phenotype with the $(TA)_7TAA/(TA)_7TAA$ Genotype.




I, II, III = generations in family * = genetic and biochemical data available

□ male

○ female

▨ ◍ homozygotes for the $(TA)_7TAA$ allele

◐ heterozygotes for the $(TA)_7TAA$ and $(TA)_6TAA$ alleles

(13) = total serum bilirubin

(18-25) = elevated total serum bilirubin

FIG. 3

AP1

-611 GTGAGTCTGGCTCACCTCATGGCGCGTGGCTCGTGTGGTGGGCTCTGCTGCAGCCTCCAA

Sp1

-541 GACACCACACTGTGCTGGACTCAATAAATAATGTTGGACGAAGGAATGAAACACATGATA

-491 CAAGTGAGCAGGCAGTACCGGGGGGAGCTGTGGAGTGGGCACTCTTACAGGTTTCCATGGC

-431 GAAAGCGGGGGGGACAGTTGTGTTCTTTTCTTTCTAAAAGGCTTTCTAAAAAGCCTTCTGT

-371 TTAATTTCTGGAAAAGAAGCCTAACTTGTTCACTACATAGTCGTCCTTCTTCCTCTCTGG

AP3

-311 TAACACTTGTTGGTCTGTGGAAATACTAATTTAATGGATCCTGAGGTTCTGGAAGTACTT

CLBP AP1

-251 TGCTGTGTTCACTCAAGAATGTGATTTGAGTATGAAATTCCAGCCAGTTCAACTGTTGTT

-191 GCCTATTAAGAAACCTAATAAAGCTCCACCTTCTTTATCTCTGAAAGTGAACTCCCTGCT

AP1 HNF1

-131 ACCTTTGTGGACTGACAGCTTTTTATAGTCACGTGACACAGTCAAACATTAA

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-11 GCAAAGGCGCCATGGCTGTG

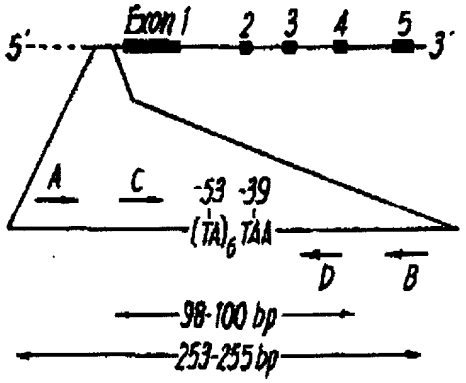


FIG. 4

# Declaration and Power of Attorney For Patent Application
## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
DRUG TRIAL ASSAY SYSTEM,
the specification of which is attached hereto unless the following box is checked:

☒ was filed on 03 March 1997 as
United States Application Number or PCT International Application Number PCT/GB97/00577
and was amended on 03 April 1998 and 01 September 1998 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

| Prior Foreign Application(s) | | | Priority Not Claimed |
|---|---|---|---|
| 9604480.5 | GB | 01 March 1996 (01.03.96) | ☐ |
| (Number) | (Country) | (Day/Month/Year Filed) | |
| 9605598.3 | GB | 16 March 1996 (16.03.96) | ☐ |
| (Number) | (Country) | (Day/Month/Year Filed) | |

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

| | |
|---|---|
| _____ | _____ |
| (Application Number) | (Filing Date) |
| _____ | _____ |
| (Application Number) | (Filing Date) |

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

| | | |
|---|---|---|
| (Application Number) | (Filing Date) | (Status - patented, pending, abandoned) |
| (Application Number) | (Filing Date) | (Status - patented, pending, abandoned) |

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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| Andrew L. Ney | Reg.No. 20,300 | Christopher R. Lewis | Reg.No. 36,201 | Christopher J. Dervishian | Reg.No. 42,480 |
| Kenneth N. Nigon | Reg.No. 31,549 | Louis W. Beardell, Jr. | Reg.No. 40,506 | Jack J. Jankovitz | Reg.No. 42,690 |
| Kevin R. Casey | Reg.No. 32,117 | Rocco L. Adornato | Reg.No. 40,480 | | |
| Benjamin E. Leace | Reg.No. 33,412 | Jacques L. Etkowicz | Reg.No. 41,738 | | |
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of second joint inventor, if any (given name, family name) _____

Second Inventor's signature _____ Date _____

Residence _____

Citizenship _____

Post Office Address _____

☐ Additional inventors are being named on separately numbered sheets attached hereto.

# Declaration and Power of Attorney For Patent Application
## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
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| | | |
|---|---|---|
| _____ | _____ | _____ |
| (Application Number) | (Filing Date) | (Status - patented, pending, abandoned) |
| _____ | _____ | _____ |
| (Application Number) | (Filing Date) | (Status - patented, pending, abandoned) |

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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| Allan Ratner | Reg.No. 19,717 | Robert L. Andersen | Reg.No. 25,771 | Joshua L. Cohen | Reg.No. 38,040 |
| Andrew L. Ney | Reg.No. 20,300 | Christopher R. Lewis | Reg.No. 36,201 | Christopher J. Dervishian | Reg.No. 42,480 |
| Kenneth N. Nigon | Reg.No. 31,549 | Louis W. Beardell, Jr. | Reg.No. 40,506 | Jack J. Jankovitz | Reg.No. 42,690 |
| Kevin R. Casey | Reg.No. 32,117 | Rocco L. Adornato | Reg.No. 40,480 | | |
| Benjamin E. Leace | Reg.No. 33,412 | Jacques L. Etkowicz | Reg.No. 41,738 | | |
| James C. Simmons | Reg.No. 24,842 | Eric A. Dichter | Reg.No. 41,708 | | |

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Full name of second joint inventor, if any (given name, family name) _____

Second Inventor's signature _____ Date _____

Residence _____

Citizenship _____

Post Office Address _____

_____

☐ Additional inventors are being named on separately numbered sheets attached hereto.